

Incidence and Histopathological Study of *Xanthomonas axonopodis* pv. *vesicatoria* (Doidge) Dye in Chilli (*Capsicum* spp.) Seeds in Western Rajasthan

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ABSTRACT

Bacterial leaf spot is a destructive disease in Rajasthan caused by *Xanthomonas axonopodis* pv. *vesicatoria* (Doidge) Dye. A field and market survey was conducted for the study of incidence and location. Dry seed examination of 103 seed samples of chilli (*Capsicum* spp.) belonging to 16 districts of Rajasthan that revealed a 10–100% incidence of *Xanthomonas axonopodis* pv. *vesicatoria* (XAV) on Tween-80 medium. Two naturally infected seed samples of chilli carrying a 100% incidence of XAV were selected and categorized into asymptomatic (06.25–94.25%), moderately discolored (01.75–42%), and heavily discolored (01.25–27.75%) seeds. The heavily discolored seeds showed shriveled, water-soaked symptoms on their surface, and on bisecting such types of infected seeds, the embryo and endosperm showed necrosis and browning. The pathogen was found confined to the outer seed coat layer, particularly at the ramment of funiculus in a few asymptomatic seeds. In moderately discolored seeds, the pathogen was found in the seed coat and the space in between the seed coat and the endosperm. It colonized all the seed components, including embryo and endosperm, in heavily discolored seeds. The pathogen caused necrosis, the formation of lytic cavities, a reduction in cell contents, and aggregation of the bacterial cells. The pathogen was found to be extra-as well as intra-embryonic.

Keywords: Chilli, Bacterial leaf spot, Seed discolorations, *Xanthomonas axonopodis* pv. *vesicatoria*, Seed-borne bacterial disease, histopathology.

1. Introduction

Bacterial leaf spot (BLS) disease of sweet pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) is caused by *Xanthomonas axonopodis* pv. *vesicatoria* (XAV) and has been reported from several countries in eastern and southern Africa, including the United States, Ethiopia, Kenya, Malawi, Mozambique, and South Africa (Anonymous, 1996; Black et al., 2001).

XAV isolation on semi-selective media including Tween B confirmed symptoms on tomato and sweet pepper fruit (McGuire, Jones, and Sasser, 1986; Jones et al., 2000). In India, bacterial leaf spot disease was first reported in Pune, Maharashtra in 1948 by Patel et al. in chilli. It is a major disease in tropical and subtropical climates and also appears on green fruits. In Rajasthan, the disease caused a 7.5 to 16.6 percent loss in the yield of fruits (Shekhawat and Chakravarti, 1977). The pathogen required a temperature range of 22–34°C with high humidity for maximum infection (Shekhawat and Chakravarti, 1976).

Xanthomonas axonopodis pv. *vesicatoria* (Doidge) Dye (syn: *Xanthomonas campestris* pv. *vesicatoria*) (XAV) causes bacterial leaf spot (BLS) diseases on peppers and tomatoes. It is a gram-negative, rod-shaped bacterium that attacks and produces symptoms on all the aerial parts of the plant as leaf spots, fruit spots, and stem cankers (Anonymous 1996, Thieme, Frank et al. 2005, Anonymous 2022). The BLS disease occurs universally throughout the world where conditions are relatively warm and moist reducing the quality and nutritive value for sale fresh and also for processing. When epidemics begin early in the crop (before flowering), losses in marketable fruits may be more than 50% (Pohronezny et al., 1992; Anonymous, 2022). The pathogen was found to be seed-borne (10–15%) and also subsists on infected plant debris, weeds, and volunteer tomato plants (Jones et al., 1986). The incidence was less than 5% and persisted from one season to the next in crop debris or on weed hosts (Ravinkar, Demsar, and

Dreo, 2001). Leaves with irregular necrotic spots and a halo were collected from a greenhouse in El-Minia, Egypt. Many researchers have reported that the bacterial pathogen is seed-borne in chilli (Neergaard, 1977; Bradbury, 1986; Richardson, 1990).

In the present study, the incidence and histopathology of pathogens in seed samples of chilli from Rajasthan state were studied.

2. Materials and Methods

One hundred and three seed samples of chilli collected from 16 districts of Rajasthan were subjected to dry seed examination, the standard blotter method (Anonymous, 1985), and the Tween-80 agar medium plate method (Lelliot and Stead, 1987, Saettler et al., 1989). In dry seed examination, seed samples were categorised into asymptomatic, moderately discoloured, and heavily discoloured seeds. The degree of discolouration, signs, shape, size, and outgrowths on the seed surface were studied. All the seed samples were incubated on Tween-80 medium to record the percent incidence of the pathogen in the seed samples. The isolates of the bacterium were subjected to confirmative tests of identification (Schaad, 1988).

The cultures were maintained on nutrient agar (NA) medium and pure colonies after 72 h of incubation at 30°C. Typical bacterial colonies from seeds were raised on YDC (Schaad and Kendrick, 1975) agar plates. The pure bacterial colony was subjected to various tests, namely gram's staining, KOH solubility test, levan formation, oxidase test (Kovac's, 1956; Hildbrand and Scroth, 1972), potato soft rot test, nitrate reductase test (Fahy and Persley, 1983), arginine dihydrolysis, gelatin hydrolysis test, hypersensitivity test in tobacco and pathogenicity tests (Lelliot and Stead, 1987) for the identification of the bacterial species. For all the tests, 24-48 h old cultures (Lelliot and Stead, 1987) and bacterial suspensions (Kiraley, Klement, Solymosy and Vörös, 1970) were used. Bacteria that were found by different methods were tested for pathogenicity on the host plant and other plants (Schaad, 1988).

Two seed samples of chilli naturally infected with XAV (ac nos. Ca-1234 and Ca-1227) with a 100% incidence as demonstrated on Tween-80 media were chosen for histopathology examinations. The observations were made using a serial microtome and hand-cut sections stained with saffranin and fast green combinations (Johanson, 1940).

3. Results and discussion

Out of one hundred three seed samples of chilli, 85 seed samples were found infected with a pathogen (Table 1). In dry seed examination, seed samples of chilli were categorised into asymptomatic, moderately discoloured, and heavily discoloured seeds (Fig.1A). The discoloured seeds showed shrivelling, water-soaked, translucent areas and bacterial ooze forming a crust-like growth on the seed surface. The seed surface of asymptomatic seeds looked healthy with the ideal colour of seed coat. The seeds were much discolored, and when they were cut in half (bisect), they found that the embryo and endosperm had necrosis and browning.

The bacterium colony isolated from various seed samples produced convex to domed, circular, entire, yellow, mucoid, shiny, and raised colonies on YDC agar medium and were identified as being of *Xanthomonas axonopodis*

pv. *vesicatoria* (Fig.1B). The incidence was studied on Tween-80 and YDC agar medium. On Tween-80 medium, XAV appears as circular, raised, yellow colonies surrounded by a zone of white crystals of calcium salt of fatty acids released from tween by lipolytic enzymes. The isolates were gram-negative, KOH solubility test positive, levan negative, lipase activity positive, Kovac's oxidase negative or weak, nitrates were not denitrified or reduced but catalase positive, starch hydrolyzing, gelatin hydrolyzing, arginine variable, and no rotting of potato tissue occurred. The pathogen induced a positive hypersensitivity reaction on tobacco leaves after infiltration. The colonies were smooth, circular, butyrous, or viscid, usually yellow (xanthomonadins) in colour, but a few non-pigmented strains also occurred on nutrient agar medium.

Table 1. District-wise occurrence and incidence of various bacterial species in chilli in SBM and on various semi-selective media

S. No.	District	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
		UT	PT	TWEEN-80
1.	Ajmer	2(34, 84)	2(24,74)	2(70, 100)
2.	Alwar	3(30-48)	3(28-38)	3(70-80)
3.	Bharatpur	7(24-56)	7(18-48)	7(50-80)
4.	Bhilwara	2(32,36)	2(30, 30)	2(60, 60)
5.	Bundi	1(36)	1(32)	1(90)
6.	Churu	1(40)	1(30)	1(60)
7.	Dausa	6(22-50)	6(18-36)	6(40-100)
8.	Hanumanagarh	3(18-44)	3 (18-38)	3(80-80)
9.	Jaipur	30(12-62)	30(8-62)	32(10-100)
10.	Jodhpur	5(18-68)	5(18-64)	5(50-100)
11.	Karoli	2(28, 30)	2(24, 28)	2(50, 50)
12.	Kota	2(26, 32)	2(24, 24)	2(50, 80)
13.	Sikar	2(50, 56)	2(44, 48)	2(100, 100)
14.	Sawai Madhopur	6(18-64)	6(12-50)	6(30-100)
15.	Tonk	3(34-54)	3(22-38)	3(70-100)
16.	Udaipur	8(22-38)	8(20-32)	8(30-100)
	Total	83 (12-84)	83 (8-74)	85 (20-100)

3.1. Incidence of the pathogen

The pathogen was recorded in 83 seed samples in untreated (12–84%) and pre-treated (08–74%) in the standard blotter method (SBM). The incidence of pathogens on Tween-80 medium was 10–100% in 85 seed samples out of 103 collected samples. The seed samples of Alwar (70-80%), Bharatpur (50-80%), Dausa (40-100%), Jaipur (10-100%), Jodhpur (50-100%), Sawai Madhopur (30-100%), Tonk (70-100%), Karoli (30-90%), and Udaipur (30-100%) districts revealed relatively high incidence of the pathogen (Table 1).

3.2. Histopathological studies

Both the selected seed samples of chilli naturally infected with XAV (100%) were selected for histopathological studies. In sections of asymptomatic seeds, the aggregation of the bacterial cells was confined to the ramement of funiculus (hilum), outer and inner layers of seed coat in both the samples studied (Figs.1, 2). The pathogen also colonised the inner layer of the seed coat.

The asymptomatic seeds were dull ochre in colour, often C-shaped or angular in shape, flattened. Water-soaked translucent spots on the surface of shrivelled discoloured seeds varied from light brown to black. Seeds that were heavily discoloured had dark brown to black discolouration and blotches on the surface shrivelled and were decreased in size (Fig.1 A). When discoloured seeds were bisected, dark brown coloured embryos were discovered, in contrast to asymptomatic seeds. Seed-borne bacterial infections cause seed discolouration in pea and reduce yields (Verma and Agrawal, 2018). Microtome investigations were performed on both selected seed samples from each category to determine the bacterium's location.

Moderately discoloured seeds reveal the pathogen colonisation of most of the seed components. Bacterial cells and clumps were found in the funiculus ramements (hilum), the outer layer of seed coat, the inner layer of seed coat (Fig.1C), and the endosperm (Fig.1D). In endosperm, seeds reveal bacterial colonies and lytic cavities. The pathogen was localised in all seed components, including the embryonal axis, and in between the endosperm and embryonal axis in a few seeds in both samples.

Bacterial cell aggregation and clumping were observed in heavily discoloured seeds at the funiculus ramement, outer and inner layers of the seed coat, endosperm, and embryo (Fig.1E). In some seeds, the endosperm cuticle was not intact; depletion of cell contents, formation of lysogenous cavities, aggregation of bacterial cells, and necrosis were observed (Figs.1F & G). The pathogen was also found aggregated in between the seed coat layers and endosperm and in the cotyledons (Fig.1H, I, Fig.2). On sectioning, some seeds showed deformed endosperm and brown to black embryos. In heavily infected seeds, lytic cavities formed due to the disruption of cells were quite frequent in the cotyledonary tissue. The cotyledons also showed necrosis.

Seeds with discolorations were found to be related with pathogens in the current study. In bean halo disease caused by *Pseudomonas phaseolicola*, shrivelled seeds revealed brown discolouration of seeds (Neergaard 1977). In sunflowers, *P. syringae* has caused discoloured seeds with water-soaked transparent regions on the seed surface (Godika et al., 2000). It has been found that *Xanthomonas campestris* pv. *campestris* causes brown, pinkish discoloration in mustard (Sharma et al., 1992) and *Xanthomonas cajani* pv. *cajani* causes brown, pinkish

discoloration in pigeon pea (Sharma et al., 2001), brown discoloration caused by *X. a. pv. vesicatoria* in brinjal (Sharma and Sharma, 2014).

XAV infection in chilli seeds had an adverse influence on seed quality, causing discolorations, shrivelling, and water-soaked symptoms. Symptoms induced by *Xanthomonas campestris* in cow pea (Kishun, 1987), *Xanthomonas campestris* pv. *campestris* in rape and mustard (Sharma et al., 1992), pigeon pea (Gaikwad and Kore, 1981, Sharma et al., 2001), *Ralstonia solanacearum* in tomato (Sharma and Agrawal, 2010) and *Xanthomonas axonopodis* pv. *vesicatoria* in chilli (Sharma, 2007).

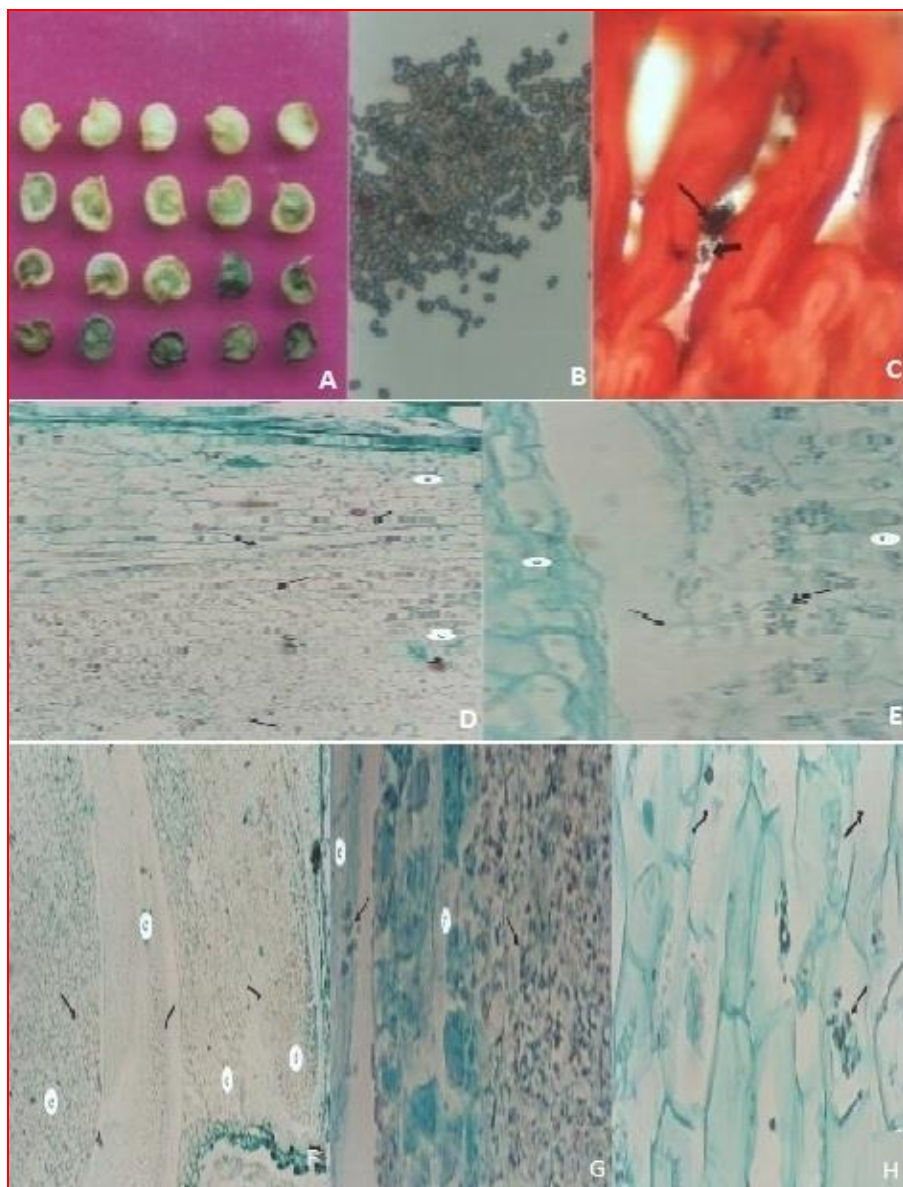


Fig.1. Histopathology of chilli seeds naturally infected with *Xanthomonas axonopodis* pv. *vesicatoria*

A- Seed categories in dry seed examination, asymptomatic (uppermost layer), moderately discoloured and heavily discoloured seeds (last two rows), B- Cells of bacterium on Gram's staining X 1000, C- Part of L.S. of moderately discoloured seed showing cells and clumps of the pathogen (←) in the outer layer of testa of seed coat X 1000, D- Part of L.S. of moderately discoloured seed showing heavy colonisation of pathogen (←) in endosperm (e) and cotyledons tissue (c) with aggregation of bacterial cells and depletion of cell contents X 250, E- Part of Fig. D

enlarged, showing the bacterial cells (←) in space in between endosperm (e) and cotyledon (c) (←) with the lysis of host cells X 1000, F- Part of L.S. of heavily discoloured seed showing the bacterial cells (←) in endosperm (e), cotyledons (c) and radicle (r). Note the necrosis and lysis of host cells X 250, G- A part of L.S. of heavily discoloured seed showing the presence of pathogen (←) in the space in between endosperm (e) and radicle (r). Note the heavy aggregation of the pathogen and depletion of cells contents X 1000, H- Part of L.S. of heavily discoloured seed showing the cells of the pathogen (←) in endosperm cells. Note the depletion of cells contents X 1000.

The bacterium was found in the outer layer of the seed coat's testa in this investigation. *X. campestris* was identified on the seed coat surface, according to Neergaard (1977), although those that cause vascular or systemic infection are commonly found in the seed coat and other tissues of the seed. The bacteria have been found somewhere under the seed coat in cabbage (Bandyopadhyay and Chattopadhyay, 1985), rape and mustard (Sharma et al., 1992), pigeon pea (Sharma et al., 2001, 2002), tomato (Sharma and Agrawal, 2010), brinjal (Sharma and Sharma, 2014) and black gram (Jain, Sharma and Agrawal, 2022).

Cells of XAV were found to be restricted to the area between the seed coat, endosperm, radicle, and hilum region in moderately and extensively discoloured seeds including aggregates of bacterial cells in the micropylar area in the current investigation. Bacteria on the seed surface can cause systemic or vascular infection, and they are often detected in the other seed tissues and seed coat layers (Skoric, 1927, Neergaard, 1977, Singh and Mathur, 2004). XAV is thought to penetrate chilli seeds through the funiculus and micropyle opening. Similarly, it was reported in the previous studies that bacterial infections have been shown to penetrate through micropyle (Skoric, 1927), wounds (Khristov, 1968), stomata (Tabei, 1967, Fukuda et al., 1990), funiculus (Naumann, 1963) or mechanical damage (Tabei et al., 1989, Agrios, 2005).

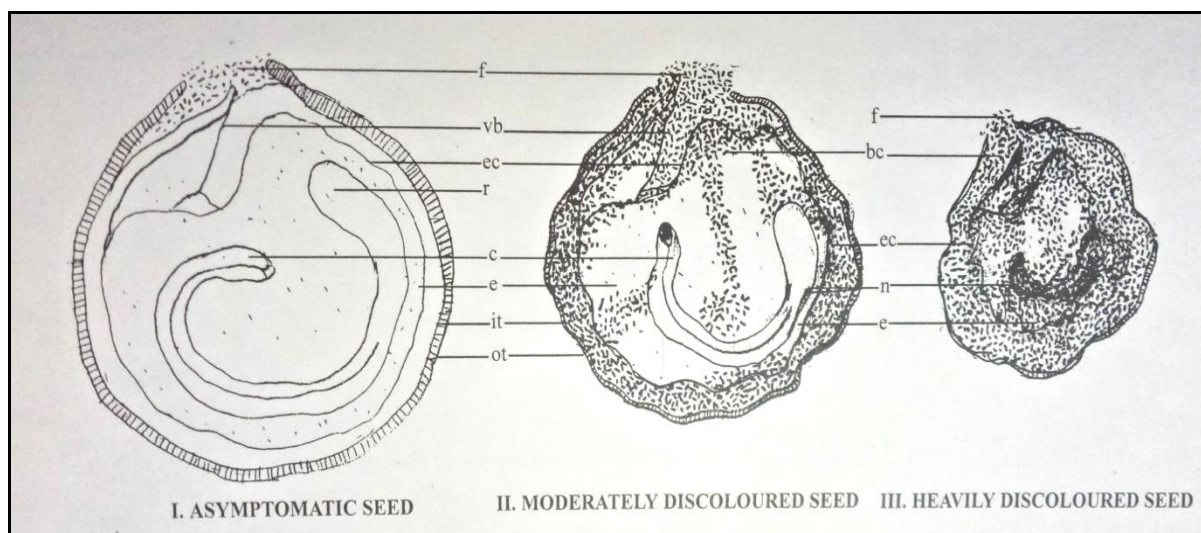


Fig.2. Semi-diagrammatic presentation of the location of *Xanthomonas campestris* pv. *vesicatoria* in naturally infected chilli seeds

bc- bacterial cells c- cotyledons it- inner layer of testa n- necrosis

e- Endosperm ec- endosperm cuticle ot- outer layer of testa r- radical

f- Remnant of funiculus h- hypocotyls vb- vascular bundle of funiculus

The bacteria were discovered in this investigation to be connected with the seed coat, particularly in the hilar area. This might be related to gas exchange and water transfer via the funiculus during seed formation, according to Verma (1990). As it is previously reported, the development of cells or clusters of bacterial cells in the hilar area showed pathogen entry via funiculus (Cook et al., 1952). The bacterium *Xanthomonas campestris* pv. *phaseoli* produced common and fuscous blight in *Phaseolous* spp. and *Dolichos lablab*, and it was found in both the seed and on seed coat (Mortensen, 1994a).

Due to infection, the infected seeds displayed cell necrosis, lysis, disintegration, or a decrease in cell contents. In pea, the raphe of seeds with vascular elements provides a good entry point for *Pseudomonas syringae* (Verma and Agarwal, 2018). The bacteria is present in soft parenchymatous tissue's inter- and intracellular spaces and induces cell lysis. By bacterial mass adhering to the funiculus cavity, the micropylar infection begins through the funiculus. *Pseudomonas syringae* pv. *lachrymans* infection of cucumber seeds begins intracellularly and intercellularly in the funiculus (Wiles and Walker, 1951, Kritzman and Zutra, 1983). In artificially infected plants, the *X. c.* pv. *campestris* penetrates in cabbage seeds via funiculus (Cook, Larson, and Walker, 1952). Infestation of *X. a.* pv. *phaseoli* in bean seed occurs in the micropyle and/or funiculus (Zaumeyer, 1930). According to Cook et al. (1952), the development of cells or clusters of bacterial cells around the hilum area indicated pathogen penetration through the funiculus.

XAV was identified extra and intra embryonal in chilli seeds in this investigation, colonising the outer layers of seed coat in asymptomatic seeds (healthy appearing seeds) and up to the embryo in symptomatic seeds. Internal infection was reported in chalazal halves and micropyle of the seed coats, as well as in embryo rarely (Tennyson, 1936; Brinkerhoff and Hunter 1963, 1964).

Internally and externally on the seed, *X. c.* pv. *malvacearum* is found. It is found in the chalaza, the micropylar end of the seed coat, and the embryo of the seed (Brinkerhoff and Hunter, 1963; Hunter and Brinkerhoff, 1964). Externally and internally up to endosperm, *X. c.* pv. *glycines* and *X. oryzae* pv. *oryzae* were detected (Fang et al., 1956, Srivastava and Rao, 1964, Groth, 1983, Mukerjee and Singh, 1983). *Pseudomonas syringae* pv. *phseolicola* is detected in the hilum area, the surface of cotyledons, and the embryo of badly infected bean (*Phaseolus* sp.) seeds (Taylor et al. 1979).

In the field visit during this study, it was seen that several other crops such as tomato, brinjal, okra, wheat, and barley, cabbage, and weed plants such as *Chaenopodium*, *Amaranthus*, *Achyrenthes*, *Medicago*, and *Asphodilus* were observed. It is an investigation that the pathogen survives on tomato and pepper plants, seeds, and debris from infected plants but it cannot live in the soil for more than a few weeks. The bacterium can be found in association with wheat roots and some weed species, which are both considered sources of inoculum as well as diseased tomato and pepper plants (Neergaard, 1977, Pohronezny, Hewitt, Infante and Datnoff, 1992, Ravinkar, Demsar and Dreo, 2001, Anonymous, 2022). In cold climates, XAV infection is mostly caused by contaminated seed material, both on and inside of seeds (Agrios, 2005). It means the survival of pathogens on seeds will infect the cotyledons at the time of the growing seedling as it emerges from the seed coat. Such internally infected seeds will produce diseased plants from the point of germination. In the present investigation, the bacterium was found associate with floral bud and persistant calyx in transmission studies. Similarly in seed of *Capsicum annuum* by isolating the *Xanthomonas*

vesicatoria in cortical and vascular tissues of pedicel, ovary (Crossan and Morehart, 1964); *Acidovorax citrulli* (causes bacterial fruit blotch of cucurbits) was observed in the cotyledons of pistil-inoculated seeds and perisperm-endosperm layers (Dutta, Avci, Hahn and Walcott, 2012), tomato seeds (Sharma, 2007), *X.c* pv. *campestris* in mustard and rape seeds (Sharma et al., 1992), and pigeon pea (Sharma et al., 2001). The bacterium *P. s. pv. lachrymans* found in the embryo and confined to the outermost layers of the radicle (Nauman, 1963).

4. Conclusions

The bacterium, *Xanthomonas axonopodis* pv. *vesicatoria*, is a seed-borne pathogen that occurred in the various components of this study. The bacterium was found to be extra-as well as intra-embryonic in chilli seeds. It was confined to the outer layer of seed coat and funiculus in asymptomatic seeds and seed coat, in the inner layer of testa, endosperm, and embryo in moderately and heavily discoloured seeds. Bacterial cells were found in large numbers at the funiculus ramanents. This suggests that this area could be a way for bacteria to get into the seed and infect it, which could lead to a systemic infection, as has been reported.

5. Future Recommendations

The seed discolorations help in the screening of healthy and infected seeds that may be helpful to the companies and industries based on the chilli seed. The bacterium is reported in the various seed components in this study. The entry point of bacteria is the natural opening in the seed coat. It will be helpful to scientists working in the fields of plant pathology, seed technology, and other relevant fields. The study will benefit the management strategy of the diseases. To know more in detail about the penetration of bacteria in the seed, advanced techniques like SEM, TEM, etc. will be beneficial and required.

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The authors declare no competing financial, professional, or personal interests.

Consent for publication

The authors declare that they consented to the publication of this research work.

Authors' Contributions

All authors equally contributed to research and paper drafting.

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